REMARKS

Reconsideration and allowance in view of the following remarks are respectfully requested. Claims 1, 4, 5, 12, 15 and 16 are presently pending in this application and stand rejected.

Claim Rejections - 35 USC § 103

On pages 3-4, in numbered paragraph 6, of the Official Action, the Examiner rejects claims 1, 4-5, 12 and 15-16 under 35 USC 103(a) as being unpatentable over Nakafuka et al. (J. Neuroscience Res. 41:153-168, 1995) in view of Weiss et al. (U.S. Patent 5,851,832). In this rejection, it is the position of the Examiner that claims 1, 4-5, 12 and 15-16 are obvious over the combination of the teachings of the cited references.

On pages 4-5, in numbered paragraph 7, of the Official Action, the Examiner rejects claims 1, 4-5, 12 and 15-16 under 35 USC 103(a) as being unpatentable over Nakafuka et al. (J. Neuroscience Res. 41: 153-168, 1995) in view of Weiss et al. (U.S. Patent 5,851,832) and further in view of Eilers et al. (Nature 340: 66-68, 1989) and/or Evans et al. (Science 240: 889-895, 1988). In this rejection, it is the

position of the Examiner that claims 1, 4-5, 12 and 15-16 are obvious over the combination of the teachings of the cited references.

These rejections are respectfully traversed. It is respectfully submitted that the combination of the teachings of the Nakafuku et al. with those of Weiss et al. would not result in the disclosure of the present invention for the following reasons.

Nakafuku et al. used the same myc-estrogen receptor fusion protein ("myc-ER") of Eiler et al. as the present invention to obtain a neural cell culture. Contrary to the Examiner's reading, however, Nakafuku et al. did not obtain a stable neural stem cell lines. They obtained unstable neural stem cell cultures. As clearly stated in the present application, the novelty of the invention is to obtain a stable cell line where the stability is defined by being able to culture beyond the cell's natural senescence point without changes in the cell's quantitative ability to differentiate into multiple lineages. Thus, in the present application, we state:

> The mitotic capacity of CNS stem cells, however, is finite. With the previous culture conditions, it had been difficult to expand CNS stem cells beyond

about 30 cell doublings at which point a majority of the cells have lost their capacity for neuronal differentiation and further expand as glial progenitors....
Here, we disclose that consititutive activation of c-myc protein in CNS stem cells prevents their spontaneous differentiation...and increases the mitotic capacity over 60 cell-doublings. This procedure thus yields more than a 10^{18}-fold expansion of CNS stem cells" (page 3, lines 3-20 of the present application).

The present application also states:

With continuous passage, CNS stem cells gradually lose their capacity to differentiate into neurons, thus becoming glial progenitors. The conditions which accelerate this process include high cell density during proliferation, poor attachment of the cells on extracellular matrix coated surface, and exposure to glia-promoting factors such as CNTF (ciliary neurotrophic factor), LIF (leukemia inhibitory factor), BMPs (bone morphogenic factors) and serum. In order to overcome this instability of neuronal differentiation capacity of CNS stem cells, we have introduced into the cells a cellular proto-oncogene, c-myc, whose activity can be regulated by the presence or absence of an extracellular molecule, β-estradiol." (page 10, lines 8-16 of the present application).

Therefore, the innovative concept and the utility of using conditionally active c-myc expression is to overcome the natural senescence of the neural stem cells imposed by cell-intrinsic, spontaneous differentiation mechanisms, which result in neural stem cell lines stable for at least 60 cell doublings.

This concept and the results are far from the teachings of Nakafuku et al. Thus, Nakafuku et al. state:

> Thus, this *mycer* construct is useful for examining the role for *c-myc* in neural stem cells....
> Thus, either growth factors or oestrogen, which activates *c-myc*, in the culture medium acted as a mitogen, whereas the combined effect of both factors was much stronger than that of single factors. Furthermore, both *c-myc* activation and bFGF also appeared to be required for maximal differentiation of MNS-57 cell into neurons and glia. (page 154, left column, first paragraph, lines 6-22, of Nakafuku et al.).

Therefore, the study concludes that c-myc and bFGF are necessary for both fast proliferation of neural precursor cells (page 159, Fig. 3, of Nakafuku et al.) and their differentiation into neurons (page 162, TABLE I, of Nakafuku et al.). This is in sharp contrast to the present

invention where both bFGF and c-myc are removed to affect the stem cell differentiation. Thus, the scope and substance of the study by Nakafuku et al. do not teach anything about the problems of instability during prolonged culture of neural stem cells, do not suggest any solutions toward such problems, and lead to conclusions regarding the utility of bFGF and c-myc that are contrary to the facts of the present invention.

Moreover, the one cell line, MNS-57, reported by Nakafuku et al. is not a stable cell line as defined in the present invention. The authors never demonstrate a serial expansion of MNS-57 for long-term. Rather, they show a short-term expansion for 6 days only (for example, page 159, Fig.3, of Nakafuka et al.). The scale of the culture is also quite small, starting with $1x10^4$ cells and ending with about $3x10^6$ cells per culture (page 159, Fig.3). What a skilled reader can infer from this is that a single cell has been expanded to about 10^6 cells, which is equivalent to about 20 doublings.

However, the cells expanded even at this stage are not multipotential neural stem cells as revealed in TABLE I, page 162 of the cited reference. Nakafuku et al. show that

when the cells grown under any culture conditions are differentiated, only 0.1-0.3% of the cells have retained the intrinsic ability to differentiate into neurons (lines 1 and 5, TABLE I, page 162). In other words, only 0.1-0.3% of the expanded cells are still multipotential after 20 doublings.

In contrast, the cells of the present invention after 54 doublings and approximately 10^{15}-fold expansion yield about 50% of the total cells as neuron in the absence of bFGF and c-myc, which is the same as the parental cells without the genetic modification.

Therefore, although Nakafuku et al. demonstrate the employment of the mycer construct of Eilers et al. for generation of a clonal neural culture, in fact, it is an unstable culture. Thus, contrary to the Examiner's assertion, a reader skilled in the art would conclude the opposite, that c-myc expression by itself or in combination with bFGF does not generate a stable, immortalized neural stem cell line.

The commercial significance of the present invention lies on two key achievements: first, a neural stem cell can be serially expanded for at least about 60 doublings

which yields 10^{18}-fold increase in cell number; and second, throughout this expansion period, the cells' differentiation property, especially to neurons, is robust and stable. To reiterate, this achievement was made based on the fundamental understanding of the factors which cause instability in neural stem cell culture and coming up with an innovative solution for the problem.

In other words, myc-ER is not needed to expand neural stem cells to the degree shown by Nakafuku et al., which is about 20 doublings. We have routinely generated neural stem cells lines up to 30 doublings without using c-myc. Thus, use of myc-ER as done by Nakafuku et al. is gratuitous. In addition, expanding a cell to 20 doublings has little commercial significance.

Thus, the present invention is not to merely grow stem cells *per se* by use of myc-ER, for which myc-ER is unnecessary, but to break through the intrinsic senescence mechanism of the cells without affecting their differentiation properties.

The Examiner further asserts that Weiss et al. teach serum-free culture of neural stem cells and that it would have been obvious to those skilled in the art to combine

with serum free culture with myc-ER use as in the Nakafuku et al. study. To reiterate, the present invention is not to merely grow stem cells *per se* by use of myc-ER, for which myc-ER is unnecessary, but to break through the intrinsic senescence mechanism of the cells without affecting their differentiation properties. With or without serum, Weiss et al. teach nothing of the intrinsic senescence mechanism at work. The concept of neural stem cell in Weiss et al. is quite different from that of the present invention. Weiss et al. believe that the defining property of a neural stem cell is self-renewal, implying that self-renewal is a perpetual process. Thus, they state that "The critical identifying feature of a stem cell is its ability to exhibit self-renewal or to generate more of itself." (column 2, lines 57-58, of U.S. Patent 5,851,832). Weiss et al. also state:

> Accordingly, a major object of the present invention is to provide a reliable source of an unlimited number of neural cells...that are capable of differentiating into neurons, astrocytes, and oligodendrocytes." (column 10, lines 30-33, of U.S. Patent 5,851,832).

Thus, contrary to the Examiner's assertion, even to those quite skilled in the art of neural stem cells, the fact that longevity of neural stem cell is finite is not obvious.

Furthermore, Weiss et al. advocate against the use of immortalization for fear of genetic instability and phenotypic limitation of a resulting cell line. Thus, they state:

> An alternative approach to spontaneously occurring cell lines is the intentional immortalization of a primary cell by introducing an oncogene that alters the genetic make-up of the cell thereby inducing the cell to proliferate indefinitely. ...they suffer from several drawbacks. First, the addition of an oncogene that alters the proliferative status of a cell may affect other properties of the cell (oncogenes may play other roles in cells besides regulating the cell cycle)....
>
> Another drawback to using intentionally immortalized cells results from the fact that the nervous system is composed of billions of cells and possibly thousands of different cell types, each with unique patterns of gene expression and responsiveness to their environment. A custom-designed cell line is the result of the immortalization of a single progenitor cell and its clonal expansion. While a large supply of one neural cell type can be generated,

> this approach does not take into account cellular interactions between different cell types. In addition...immortalization of a desired cell is not possible due to the lack of control over which cells will be altered by the oncogene." (column 9, line 31 to column 10, line 1, of U.S. Patent 5,851,832).

Therefore, Weiss et al. does not teach immortalization for any reason. The present invention demonstrates that each of these drawbacks can be overcome.

Weiss et al., in fact, does not teach serum-free culture, although the claims state that in order to distinguish over the prior art. Thus, they state that "In some cases, the medium may contain serum derived from bovine, equine, chicken and the like." (column 16, lines 21-22, of U.S. Patent 5,851,842). This is contrary to the Examiner's reading of Weiss et al.

Even if the serum-free culture of Weiss et al. and the myc-ER use of Nakafuku et al. were combined, it would not result in the present invention. Neither study realized that continuous expression of c-myc induces apoptosis and in order to ameliorate this effect, a second mitogen, notably 1% serum, is required to inhibit the myc-induced apoptosis. The cells of Nakafuku et al. are continuously

grown in a medium containing 10% fetal bovine serum and 5% horse serum (see page 155, right column, lines 14-15). Meanwhile, the cells of Weiss et al. are grown in EGF or TGFa or in combination with aFGF or bFGF but in a single medium.

As claimed in claim 1, step d), of the present invention, the order of addition of the mitogens is essential with respect to countering the apoptotic effect of c-myc with the second mitogen. Simply combining the serum-free culture of Weiss et al. with the mycer used by Nakafuku et al. would not result in the successful immortalization of neural stem cells. A second mitogen is needed to counter the c-myc-induced apoptosis. This requirement of claim 1 for a second mitogen is neither disclosed nor suggested by either cited reference.

The Examiner asserts that it would have been obvious to combine these two cited references in order to arrive at the teachings disclosed and claimed in the present invention. Nakafuku et al. report combining their mycer method with the neurosphere culture method of Weiss et al. (page 161, Fig. 5E-F; page 159, left column; page 161, Figs. 5E-F; and page 168, references to Reynolds & Weiss

and to Vescovi et al., in Nakafuku et al.). The result is an unstable, short-term culture of ill-defined neural cells as pointed out above. Thus, Nakafuku et al. were clearly aware of the study by Weiss et al.; they, however, did not really employ the serum-free culture of Weiss et al. Thus, Nakafuku et al. describe:

> The MNS-57 cells were harvested by trypsinization, and single cell suspensions were prepared at a density of 1 x 10^5 cells/ml in DF medium containing 10%FBS, 5%HS, 1μM β-E_2, and 20 ng/ml bFGF. (page 155, right column, lines 12-15).

Clearly, the authors failed to appreciate the variability of neural culture that can be caused by serum. Thus, contrary to the Examiner's assertion that combining the two cited references would have been obvious, Nakafuku et al. neither practices such combination nor suggests combining them.

At the time of the present invention, there have been several well-established practices of immortalizing neural precursor cells. Both Nakafuku et al. and Weiss et al. were knowledgeable of these practices since several of those were actually cited in both references. Thus, Nakafuku et al. state:

> To further study the nature of neural stem cells, attempts have been made to immortalize these cells (Frederiksen et al., 1988; Bartlett et al., 1988; Bernard et al., 1989; Ryder et al., 1990; Evnard et al., 1990; Redies et al., 1991; Renfranz et al., 1991; Mehler et al., 1993; also see Cepko, 1989 as a review)." (page 154, left column, lines 25-30).

Weiss et al. also state:

> This approach has been used by many groups to generate a number of interesting neural cell lines [(Bartlett et al...(1988); Frederiksen et al...1988; Trotter et al...1989; Ryder et al. 1980; Murphy et al. 1991; Almazan and McKay et al...1992]. (column 9, lines 35-41, of U.S. Patent 5,851,832).

The above-cited references from U.S. Patent 5,851,832 refer to immortalization methods distinct from using the conditional c-myc.

In spite of knowing of each other's work, neither Nakafuku et al. nor Weiss et al. teaches combining the teachings of the other. Also, neither reference provides any guidance as to why one method would be more successful than another, or suggest on what basis (such as stability of multipotentiality) someone skilled in the art could distinguish among these methods. Therefore, it is

respectfully submitted that it would not have been obvious to those skilled in the art to combine the teachings of these two cited references.

Please consider the following as further evidence of the non-obviousness of the present invention. As late as in the year 2000 (i.e., subsequent to the filing date of the present application), Villa et al. reported a study to immortalize human neural stem cell lines by investigating seven different immortalizing schemes (Villa, A. et al., Experimental Neurology 161: 67-84, 2000). A copy of the Villa et al. article is attached for the Examiner's convenience. None of the schemes disclosed in the article involved the conditionally activated c-myc approach. The authors, however, did employ the serum-free culture method of Weiss et al. More specifically, Villa et al. state:

> Serum-free, chemically defined cell culture medium (designated Human Stem Cell (HSC) medium) contained DMEM:F-12 (1:1), N2 supplements, and 1% bovine serum albumin." (page 70, left column, lines 8-11, of Villa et al.).

Therefore, contrary to the Examiner's assertion, at the time of the present invention it would not have been obvious to those ordinarily skilled in the art to combine

the serum-free culture of Weiss et al. with the mycer used by Nakafuku et al. to derive a stable neural stem cell line.

The Examiner also asserts that it would have been obvious to combine the teachings of Eilers et al. (Nature 340: 66-68, 1989) with Weiss et al. and Nakafuku et al. Eilers et al. teaches that fusing two unrelated proteins, c-myc and the ligand binding domain of estrogen receptor, creates a new functional protein in which each half maintains its original function but, when fused, results in a new activity. The Examiner cites further examples of chimeric proteins, in particular rat glucocorticoid receptor and E1A. The observations that certain combinations of DNA binding proteins with ligand binding proteins can create useful chimeric proteins have been important. The present invention certainly borrows from that concept as well as the actual construct. However, Eilers et al. used the conditionally activated c-myc to transform, not to immortalize, an already established rat fibroblast cell line. Transformation is a tumorigenesis, which is a radical change in a cell's properties. The authors were studying the role of c-myc, which was then a

newly discovered proto-oncogene, in turning a non-tumor cell line into a tumor cell line. Also, the Rat-1A cell line used by Eilers et al. is a spontaneously derived, stable cell line whose genetic make-up has not been reported. Thus, contrary to the Examiner's reading, the Eilers et al. study does not reveal or predict how and whether c-myc will be effective in primary neural cells (with the normal genetic make-up) for immortalization, which is overcoming a cell's natural senescence.

The teachings of Evans (Science 240: 889-895, 1988) on steroid hormone receptors also emphasize the structure-function relationship of these proteins, which explains why they work well as chimeras. The teachings of Evans do predict that, when combined with those of Eilers et al., other steroid hormones would substitute for the estrogen receptor portion of the mycer fusion protein, as stated in Eilers et al. and as pointed out by the Examiner. However, these teachings do not predict how c-myc might be used to overcome the intrinsic senescence mechanisms of neural stem cells; nor do they suggest that a stable neural stem cell line can be obtained by its continued expression.

The Examiner further asserts that it would have been obvious to those skilled in the art to combine the teachings of Eilers et al. and/or those of Evans with Weiss et al. and Nakafuku et al. Since other steroid hormone receptors are expected to be equally substitutable for the estrogen receptor portion of the mycer fusion protein, combining Nakafuku et al. with Eilers/Evans would be expected to result in substantially similar cultures as reported by Nakafuku et al. That is, even if c-myc is conditionally regulated by activation of a thyroid hormone receptor, for example, in the cultures described by Nakafuku et al., the result would be similarly unstable, short-term, ill-defined neural cells as described above.

Further combining such cultures with the teachings of Weiss et al. would not lead to the present invention for the reasons described above, and it would not be obvious to combine the teachings of these multiple references for the reasons presented above.

Finally, it is respectfully submitted that the teachings of Eilers et al. (Nature 340: 66-68, 1989) and Evans (Science 240: 889-895, 1988) do nothing to overcome

the above-give shortcomings of Nakafuku et al. and Weiss et al.

It follows from the above that the combination of the teachings of the cited references fails to disclose or suggest the unexpected results obtained with the methods of claims 1, 4, 5, 12, 15 and 16. It is, therefore, respectfully requested that these rejections be withdrawn and that claims 1, 4, 5, 12, 15 and 16 be allowed.

All rejections having been addressed, it is respectfully submitted that the present application is in condition for allowance and a Notice to that effect is earnestly solicited.

Should any matters remain in this application which might be resolved by interview, the Examiner is requested to telephone the undersigned at (570) 386-5744.

Respectfully submitted,

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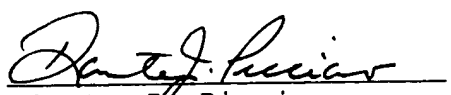
By: 

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